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Effect of Industrial Particulate Samples on Alveolar Macrophages

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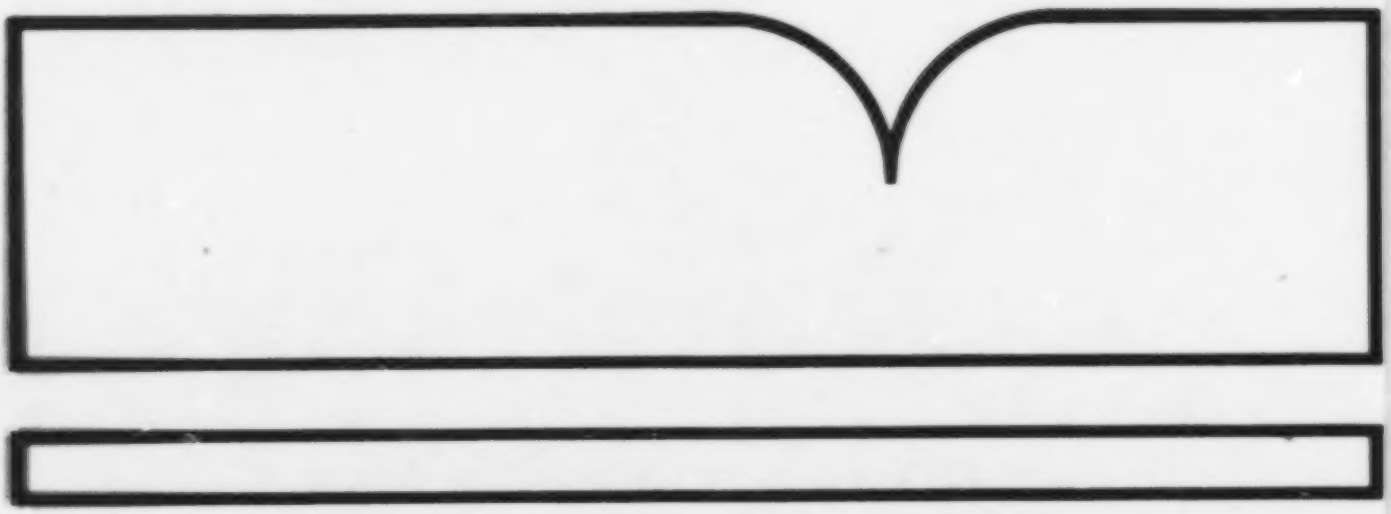

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Toxic ranking of the <3 μm size fraction of particulate samples by *in vitro* rabbit alveolar macrophage assay showed generally low to intermediate cytotoxicity for samples obtained from a steel foundry, an electric arc furnace steel plant and from four coal-fired power plants. Particulates obtained from an aluminum and from a copper smelter were highly toxic to alveolar macrophages as monitored by viability, total cellular protein and ATP levels. The two smelter samples also contained soluble components that significantly contributed to their cytotoxicity. None of the particulate samples tested were true emission or effluent samples, but were collected from in-plant control devices. Inhalation of copper smelter particle aerosols produced significant changes in more of the parameters than inhalation of the coal fly ash. Thus, the overall objective of these studies was realized by demonstrating the validity of prediction of inhalation hazard on the basis of the *in vitro* screening assay.

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EFFECT OF INDUSTRIAL PARTICULATE SAMPLES ON ALVEOLAR MACROPHAGES

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FOREWARD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This report compares the relative toxicity ranking resulting from *in vitro* exposure of complex industrial particles to alveolar macrophages with that obtained from *in vivo* aerosol inhalation exposures in intact animals. Because *in vitro* testing is both faster and less expensive, it can be a highly efficient toxicity screening assay.

F. Gordon Hueter
Director
Health Effects Research Laboratory

ABSTRACT

Toxic ranking of the <3 μm size fraction of particulate samples by *in vitro* rabbit alveolar macrophage assay showed generally low to intermediate cytotoxicity for samples obtained from a steel foundry, an electric arc furnace steel plant and from four coal-fired power plants. Particulates obtained from an aluminum and from a copper smelter were highly toxic to alveolar macrophages as monitored by viability, total cellular protein and ATP levels. The two smelter samples also contained soluble components that significantly contributed to their cytotoxicity. None of the particulate samples tested were true emission or effluent samples, but were collected from in-plant control devices.

The copper smelter dust and the fluidized-bed coal fly ash chosen on the basis of their respective high and low *in vitro* cytotoxicity were used in aerosol exposures to examine their *in vivo* effects on the pulmonary free cells, bactericidal activity and resistance to respiratory infection in mice. The results of multiple daily 3-hr exposures to 2.0, 1.0 and 0.5 mg/m^3 of the pollutants closely correlated with the *in vitro* data. Inhalation of copper smelter particle aerosols produced significant changes in more of the parameters than inhalation of the coal fly ash. Thus the overall objective of these studies was realized by demonstrating the validity of prediction of inhalation hazard on the basis of the *in vitro* screening assay.

This report is submitted by IIT Research Institute in fulfillment of EPA Grant No. R805141. Work has been completed in the period extending from March 1977 to April 1980.

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ABBREVIATIONS

AM	--	alveolar macrophages
ATP	--	adenosine triphosphate
DMSO	--	dimethyl sulfoxide
EAF	--	electric arc furnace
EC	--	exposure concentration
ESP	--	electrostatic precipitator
HBSS	--	Hanks' balanced salt solution
HEPA	--	high efficiency particulate air filter
MMD	--	mass median diameter
RAM	--	rabbit alveolar macrophage
SDC	--	sodium deoxycholate
THB	--	Todd Hewitt broth

SECTION 1

INTRODUCTION

Alveolar macrophages protect the lungs principally by phagocytosis of inhaled particles and infectious agents. Adverse effects on the activity of the alveolar macrophages can lead to increased retention of non-biological particles, as well as to impairment in the defensive capacity of the lung and consequent increased susceptibility to respiratory disease. Since resistance to infection is known to be lowered by exposure to pollutants, changes in the functional characteristics of alveolar macrophages can be used to monitor effects of such exposure in the intact animal. However, since alveolar macrophages can be obtained easily by tracheobronchial lavage and maintained in culture, they are frequently used in *in vitro* toxicology to assess the potential inhalation hazard of various substances.

The advantages of *in vitro* screening assays in terms of cost and time efficiency are well known. The rabbit alveolar macrophage test, a rapid, *in vitro* assay has been used extensively at EPA and IITRI laboratories to evaluate the relative cytotoxicity of a variety of soluble compounds and particulates (1,2,3,4,5,6,7,8). This bioassay system is capable of screening and toxicity ranking of a broad spectrum of materials and thereby identifies not only the potentially hazardous, but also the inert compounds. Based on results of the *in vitro* assays the number of samples which must be studied further *in vivo* can be significantly reduced.

The objective of the studies was to determine if *in vitro* exposure of alveolar macrophages to a series of complex industrial particles resulted in the same relative toxicity ranking as *in vivo* aerosol inhalation exposures to these particles in intact animals. The studies were designed to examine the relative toxicity of a number of particulate samples. The *in vitro* studies provided initial information on the toxicity of the particles and these data formed the basis for selection of samples for the subsequent *in vivo* investigations. The test particles used in the studies were collected from various metal manufacturing plants and coal-fired power plants directly from in-plant control devices and were not post-control device emission samples.

During the first phase of the studies, *in vitro* screening of particles using the rabbit alveolar macrophage assay was carried out. The dose response experiments provided information on the effects on viability, cell lysis, total protein and ATP levels in the macrophages and enabled the establishment of a toxic ranking of the test materials. These studies were followed by multiple daily 3-hr aerosol exposures of mice to two of the samples that had shown high (copper smelter dust) and low (fluidized-bed coal fly ash) cytotoxicity in the *in vitro* screening. The effects of inhalation exposure on the pulmonary defense system were monitored by examination of the pulmonary cellular lavage, determination of

bactericidal capacity of the lungs for inhaled radiolabeled *K. pneumoniae*, and evaluation of susceptibility to respiratory streptococcal infection. Results of these studies showed a correlation between effects of *in vitro* and *in vivo* exposures and consequently demonstrated that potential inhalation hazard of particulate substances to host defense systems can be predicted by the *in vitro* rabbit alveolar macrophage assay.

Results of the studies were summarized in a series of quarterly progress reports (IITRI Report Nos. L6093-1 through L6093-9 and L6111-10 and L6111-11). Phases of the studies were reported at the "International Symposium on Health Effects of Synthetic Silica Particulates," organized by the ASTM and the Industrial Health Foundation, November 1979, Torremolinos, Spain, and in a poster presentation at the "Symposium for Application of Short-term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures," organized by EPA, March 1980, Williamsburg, VA.

Publications resulting from the studies are:
Evaluation of potential inhalation hazards of particulate compounds. C. Aranyi, D. E. Gardner and J. Lewtas Huisingh, ASTM Journal, in press.
In vitro and *in vivo* evaluation of potential toxicity of industrial particulate effluents. Proc. Symp. Application of Short-Term Bioassays in the Fractionation and Analysis of Complex Environ. Mixtures, March 1980, Williamsburg, VA. C. Aranyi, J. Bradof, D. E. Gardner, and J. Lewtas Huisingh, in press.

SECTION 2

CONCLUSIONS

These studies were designed to determine if *in vitro* exposure of alveolar macrophages to a series of complex industrial particles resulted in the same relative toxicity ranking as *in vivo* exposure on the host defense system. The main objective was to establish a correlation between the effects of *in vitro* and *in vivo* exposures and to determine if inhalation hazard could be predicted on the basis of the *in vitro* screening.

Particles provided by the EPA were collected as baghouse samples, or from electrostatic precipitators, or by cyclone sampling train from three conventional and one fluidized-bed coal fired power plants, an electric arc furnace steel plant, a steel foundry and an aluminum and a copper smelter. None of the samples reported here were emission or effluent samples collected after the pollution control devices and were not necessarily similar in composition or toxicity to emission samples. All samples were air classified and only particles in the <3 μm size-fraction were used.

The *in vitro* effects on rabbit alveolar macrophages were monitored in dose response experiments using cell viability, total protein and ATP levels as experimental parameters. Regression analysis applied to these data showed a significant negative linear dose response relationship for each parameter in all samples, thus enabling the evaluation of their relative cytotoxicity. The results indicated that particles from a steel foundry and an electric arc furnace steel plant, and coal fly ash samples from three conventional combustion processes and one fluidized-bed system had a low to intermediate cytotoxic effect. Samples from a copper and an aluminum smelter ranked high in cytotoxicity relative to all others, with the copper smelter sample being the most toxic. In addition to the particles *per se* soluble components released from copper and aluminum smelter samples contributed to their cytotoxic effect on alveolar macrophages.

To confirm the relevance of the *in vitro* assay the inhalation hazard of these particulate samples was studied in intact animals. Mice were exposed 3 hr/day, 5 days/week for up to 4 weeks to aerosols of the copper smelter dust and of the fluidized-bed coal fly ash, i.e., particles that had shown high and low cytotoxicity *in vitro*. Aerosol mass concentrations used were 2040 and 1010 $\mu g/m^3$ for the coal fly ash and 2050, 1020 and 540 $\mu g/m^3$ for the more toxic copper smelter dust. The effects of inhalation on the pulmonary defense system were determined after 5, 10 and 20 exposures by examination of the pulmonary cellular lavage, bactericidal activity in the lungs and the resistance to experimentally induced respiratory bacterial infection. Results of these studies substantiated the *in vitro* observations by demonstrating that inhalation of copper smelter dust was significantly more deleterious (i.e. increased susceptibility to streptococcus

infection and decreased pulmonary bactericidal activity) than of the fluidized-bed coal fly ash. The major objective of these studies was accomplished by demonstrating the feasibility of predicting the potential inhalation hazard of a particulate substance on the basis of the *in vitro* alveolar macrophage screening assay.

SECTION 3

RECOMMENDATIONS

Copper smelter dust which contained 13% arsenic in addition to such other major trace metal constituents as lead, copper, iron, antimony and zinc showed high *in vitro* cytotoxicity in the rabbit alveolar macrophage assay. These *in vitro* studies also indicated that much of the arsenic can be solubilized from the particles and the leachate *per se* is toxic to alveolar macrophages. Inhalation of copper smelter dust (<3 μm aerodynamic diameter) significantly reduced the pulmonary bactericidal activity and the resistance to respiratory bacterial infection in mice. If the arsenic content is used as one basis of assessment of the health hazard of the copper smelter dust, the experimental results suggest that five daily 3-hr aerosol exposures to an equivalent of 266 $\mu g/m^3$ of arsenic resulted in significantly increased mortality rates from streptococcal pneumonia. Moreover, a significant depression of pulmonary bactericidal activity was seen after five daily exposures to 133 $\mu g/m^3$ of arsenic. These concentrations are considerably lower than the TLV of 500 $\mu g/m^3$ of arsenic.

Most of the arsenic in the atmosphere is the consequence of emissions from copper, lead and zinc smelters. At smelting operating temperatures arsenic trioxide is formed that, upon cooling, condenses on the surface of small particulate effluents. Since soluble arsenic trioxide adsorbed onto particulates of<3 μm may easily penetrate into the gas-exchange region of the lung, it can be released there. Larger aerosol particles containing arsenic in soluble and/or non-soluble form can partially be adsorbed in the upper respiratory tract and the conducting airways.

Thus, studies should be conducted to determine if particulate aerosols that contain components potentially soluble in the respiratory tract *a priori* are more hazardous than aerosols of non-soluble particulates. Furthermore studies are necessary to explain how the respiratory defense systems are affected by the form of arsenic or by the particle size of the inhaled aerosol. Since the copper smelter dusts also contain other metals such as lead, copper zinc and antimony that can potentially contribute to their toxicity, studies should be undertaken to compare the effects of inhalation of smelter dusts with those of aerosol exposures to such trace metals in a soluble form and at chemically equivalent concentrations.

The *in vivo* studies indicated increased susceptibility to respiratory infection in mice exposed to copper smelter dust particles. Further studies are necessary to determine the effects of inhalation of such toxic particulate aerosols on the cellular and humoral immune systems and thereby elucidate the changes in the immune mechanisms due to particulate pollutant-induced immunotoxicity.

SECTION 4

MATERIALS AND METHODS

PARTICULATE TEST SAMPLES

The samples collected from various stationary point sources, including fly ash (Nos. 1, 2 and 3) from coal fired power plants, and particulate by-products from a steel foundry, an electric arc furnace (EAF) steel plant and an aluminum and a copper smelter were provided by EPA's Industrial Environmental Research Laboratory (IERL) at Research Triangle Park, NC. All relevant information on these samples and inorganic analysis of some of these particulate materials was also obtained from the IERL. An additional coal combustion fly ash (No. 4) was provided by Dr. Judith Graham of the Inhalation Toxicology Branch of EPA at Research Triangle Park in cooperation with the Oak Ridge National Laboratory Chemical Repository.

- *Coal fly ash No. 1* collected as a baghouse sample from a power plant originated from high-sulphur-containing Eastern coal. No further information on place and date of collection, or chemical analysis was available.

- *Coal fly ash No. 2* was collected on March 31, 1977 by cyclone sampling train at 850°C from the second cyclone of a calcium oxide-fluidized-bed coal combustion process in an experimental demonstration plant. Because of the high collecting temperature, residual organic compounds were <0.1%. Major inorganic components identified by spark source mass spectroscopy were: Ca, Mg, Fe, Si, and S.

- *Coal fly ash No. 3* was collected as a baghouse sample from a conventional power plant. It originated from high-sulphur-containing Eastern coal. No collection data and analytical chemical information were provided.

- *Coal fly ash No. 4*, an electrostatic precipitator (ESP) hopper fly ash collected from a conventional power plant originated from high-sulphur-containing Eastern coal. It contained As, Ba, Co, Cr, Cu, Pb, Sr and Zn as some of the more prevalent trace metals and also traces of polycyclic aromatic hydrocarbons. In-depth analytical information has been published in a report prepared by Griest and Guerin (9) at the Oak Ridge National Laboratory.

- *Steel foundry particulates* were collected on May 23, 1975 as a baghouse sample from high efficiency fabric filters. Semi-quantitative analysis showed the major inorganic constituents as Fe (50%), Si (10%), Mn (5-10%) and Zn (1-3%).

- *EAF steel plant particulates* were collected on October 19 and 20, 1972 as baghouse samples at 38 to 71°C in Dacron bags. No information concerning chemical constituents was made available to us.

- *Copper smelter dust* was collected by ESP at 200°C. No organic components have been identified, but trace metals found by spark source mass spectroscopy in concentrations >1% included: Pb (18%), As (13%), Cu (8%), Fe (3%), Sb (3%) and Zn (2%).

- *Aluminum smelter dust* was collected as a baghouse sample at 120-200°C and contained 3 mg/g of extractable organic compounds, most of which were fused aromatics. No information was available on the inorganic constituents.

All collected samples were air classified and particles in the size-fraction of <3 μm were used in the experiments. Since our previous studies (1) demonstrated that the smallest particles had the most deleterious effects on alveolar macrophages (AM) *in vitro*, the *in vivo* correlation for this particle size range was of special interest.

All particulate samples were tested for mutagenicity by the Ames test (10) in our laboratory before initiation of the studies to implement the appropriate safety measures in the experimental procedures if necessary. The samples were applied as a powder, as a dimethyl sulfoxide (DMSO) extract at 500 μg/ml and in form of their supernatants after incubation at 2000 μg/ml at 37°C for 20 hr in Medium 199-in Hanks' balanced salt solution (HBSS). Test results for all samples were negative.

IN VITRO METHODS

Animals

Male albino New Zealand rabbits (1.5-2 kg) were used to obtain high yields of AM. Tha animals were quarantined for 2 weeks before the experiments and food and water was provided *ad libitum*.

Preparation of Test Suspensions

Harvesting Cells from the Lung--

Alveolar macrophages were obtained by tracheobronchial lavage. Rabbits were killed by injection of sodium pentobarbital in the marginal ear vein and the lungs were lavaged through a catheter inserted into an incision in the trachea, with consecutive 30 ml infusions of warm (37°C) saline.

Isolation and Characterization of Cells--

The AM were isolated from the lavage fluids by centrifuging at 365xg and washing in HBSS. Total cell counts were made in a hemocytometer. For determination of cellular distribution (i.e., percent of AM, polymorphonuclear leukocytes and lymphocytes) differential counts were made on smears of cells obtained in a cytocentrifuge, fixed in methanol and stained with Wright's stain. Viability of AM was determined by dye exclusion using 1% trypan blue.

Exposure to Particles--

Medium 199 in HBSS was used for incubation and exposure of the cells to the particulates. Supplements in the maintenance medium included 25mM HEPES buffer to further stabilize the pH, 100 µg/ml Gentamicin (Schering Corp., Bloomfield, NJ) and 10% heat-inactivated fetal calf serum. Separate cell and particle suspensions were prepared in the medium at twice the projected exposure concentrations and then equal volumes of the two suspensions were mixed. Serum was omitted from the particle suspension to prevent coating of the particles and was added to provide 20% concentration in the cell suspension. The final cell concentration in the test suspensions was maintained constantly at $1x10^6$ AM/ml, whereas the particle concentrations were varied to produce a change in cell viability over a wide range (20 to 90%). The test suspensions were incubated in 2.0 ml aliquots in triplicate in wells of disposable plastic cluster dishes (Falcon 3004, Falcon Oxnard, CA) placed on a rocker platform for 20 hr periods at 37°C in a humidified 5%-CO_2 atmosphere.

Measurement of Experimental Parameters

Immediately after incubation percent viability was determined by dye exclusion technique. The test suspensions were subsequently washed, centrifuged and resuspended in HBSS before monitoring total cellular protein and adenosine triphosphate (ATP) levels.

Viability--

After incubation the AM that had attached were removed by use of rubber policemen, the test suspensions were chilled and viability was determined counting 200 or 400 cells.

Adenosine Triphosphate--

Cellular ATP levels were determined in a DuPont 760 Luminescence Biometer (E. I. DuPont De Nemours & Co. Inc. Wilmington, DE) with the procedure recommended for the instrument. The assay is based on the principle that when a microsample containing ATP is injected into a suitably buffered reaction mixture of luciferase and luciferin, the peak intensity of the resulting light flash is directly proportional to the concentration of ATP. ATP was extracted from the cells by dimethyl sulfoxide (DMSO) from aliquots of the test suspension. The DMSO extracts were diluted with a specially prepared 0.01 M morpholinopropane sulfonic acid buffer to overcome the quench effect of the high concentration of DMSO in the aquenous extract for the luciferase-luciferin reaction.

Protein--

For determination of total cellular protein content, aliquots of the test suspensions were treated with 1% sodium deoxycholate (SDC), centrifuged at 10,000 xg for 15 min to remove particulates and assayed by the Lowry method (12).

Testing for solubility of Test Particles

To determine if leachable components contributed to the total cytotoxic effect, the highest exposure concentrations of the particles were incubated under identical conditions to those used in the dose response experiments but omitting the cells. After incubation the particles were removed from the media by centrifugation at 365xg, resuspended in fresh medium, and the supernatant was centrifuged at 10,000xg for 15 min and filtered through a 0.22 µm bacterial filter. The supernatant and the resuspended particles were then separately tested for cytotoxicity. A portion of the preincubated particles was tested for effect on AM viability without removing the medium containing the solubilized substances.

Experimental Design and Data Analysis

Six to nine replicate experiments were conducted for each test sample using AM obtained from separate rabbits for each replicate. Within one experiment a minimum of five exposure concentrations selected to produce a viability response over a wide range were tested in triplicate cultures using three wells of a cluster dish and three replicate assay determinations within each well for every parameter examined.

Total cellular protein values were given per 1.0 ml of the initial incubation sample. ATP levels were expressed in femtograms (10^{-15}g) of ATP per µg of total protein (experimentally determined). Changes in cellular protein and ATP levels are expressed as a percentage of the 20 hr controls. Since viability of the control AM was not affected by the incubation period, viability values were reported directly as a percentage of the total AM in the experimental sample. Linear regression and inverse prediction were used for statistical evaluation of dose reponses, and toxic ranking was established by estimating the exposure concentrations required to reduce an experimental parameter to 50% of the control value (EC_{50}). Most of this methodology has been previously published by Aranyi et al (1).

IN VIVO METHODS

Animals

Female CD_1 mice (Charles River Breeding Laboratories, Portage, MI) were used for the inhalation exposure studies. All animals were quarantined for 7-14 days before being used in the studies. During the quarantine period and throughout the experiments, mice were housed in groups of six in stainless steel shoe box cages. Food and water were provided *ad libitum*. During the exposure to the test aerosols and infectious challenge, the mice were housed individually in separate compartments of specially designed stainless steel wire cages.

Inhalation Exposures

All inhalation exposure facilities were located in rooms maintained under negative pressure relative to outside areas. The animal exposure chambers and for toxic, infectious and radioactive particulates, the aerosol generation and dilution systems as well were housed in second chamber enclosures (safety cabinet-glove box) that permitted safe handling of the animals and maintenance and monitoring of the experimental environment. The actual inhalation chambers were at all times at negative pressure relative to the outside chamber systems and the latter were negative relative to the pressure in the room. The differential pressures were continuously monitored by Magnahelic pressure gauges (Dwyer, Michigan City, IN). In case of failure in the main exhaust system an alarm (siren) alerted personnel to discontinue aerosol exposures immediately.

Animals were exposed to the experimental environment in various sized (87 to 476-liter) Plexiglas chambers that can hold for 3 hr periods up to 240 mice confined in individual compartments of wire cages. To prevent a build-up of ammonia during the exposure of animals, deotized cage boards (Upjohn Co., Kalamazoo, MI) were placed on the floor of the chambers. Temperature in the exposure chambers was maintained at 24 $\pm$ 2^{o}C at ambient humidity (<50% RH) by providing adequate airflow. Relative humidity was continuously monitored by Airguide Model 605 humidity indicators (Airguide Instrument Co., Chicago, IL) located in each chamber. An electro-hygrometer (Lab-line Instruments, Inc., Melrose Park, IL) was used to calibrate the Airguide indicators.

Exposure to Aerosols of Test Particles--

Exposure Chambers--A special inhalation exposure facility was built for use with aerosols of a copper smelter flue dust containing high levels of arsenic. The safety features provided to protect personnel from accidental exposure to the test compounds were tested by an industrial hygiene consulting firm and found in compliance with OSHA regulations for work with arsenic. Except for these safety features the animal exposure chamber and the aerosol generation and monitoring system used for inhalation exposures to the fly ash particles is identical with the one described below.

The exposure chamber housed in a stainless steel glove box is shown in Plate 1A and a schematic diagram is provided in Figure 1. The chamber consisted of two compartments; a main 476-liter compartment which permitted exposure of 240 mice and an adjacent 260-liter airlock separated by a sliding door which clamped firmly closed. The various components of the aerosol generation and dilution system i.e., the appropriate filter systems to dry and remove traces of oil and particulates from the compressed air used for dissemination and dilution, along with powder dispersing devices, dilution flasks and control gauges were also housed inside the glove box on a shelf suspended over the exposure chamber. Thus, any leakage during aerosol generation was contained and filtered along with the exhaust from the exposure chamber. To gain access to this area a series of glove ports were positioned at the upper level of the safety cabinet and a trap door built into the upper wall of the airlock served for passing the collecting filters to several sampling ports located on the top of the exposure chamber.

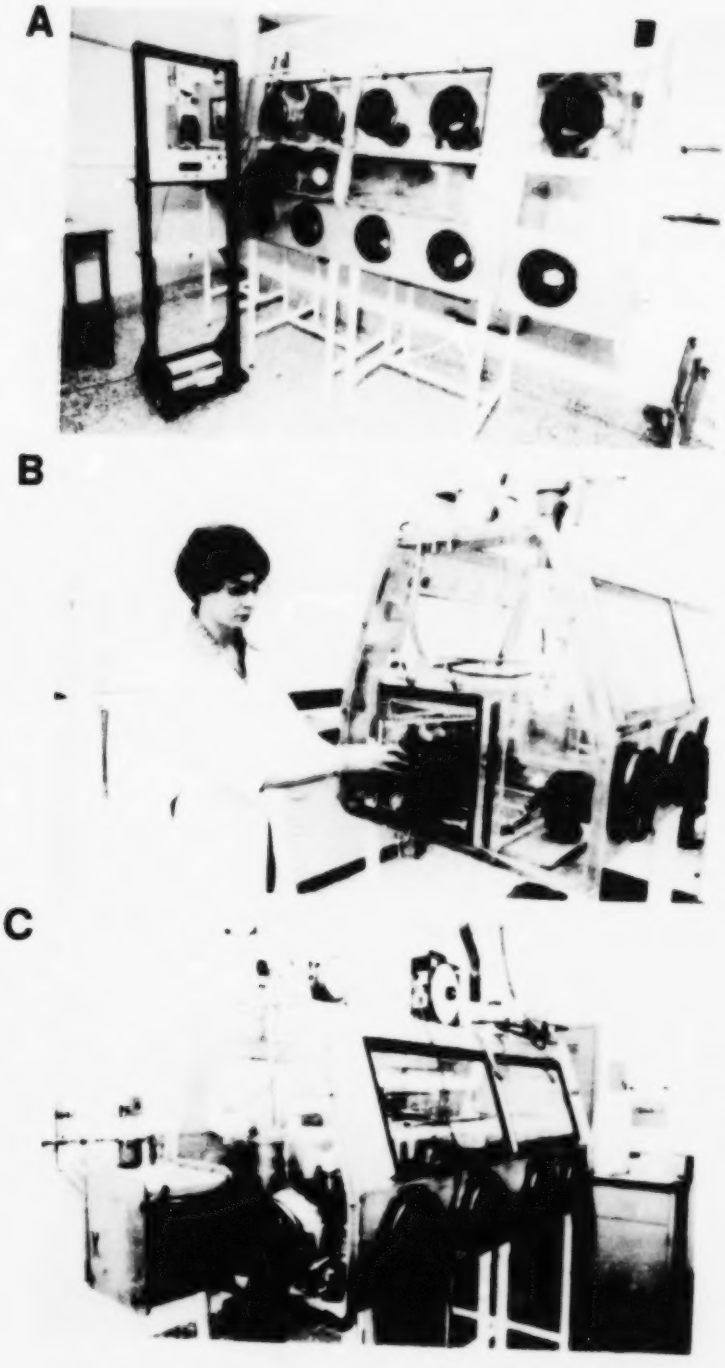
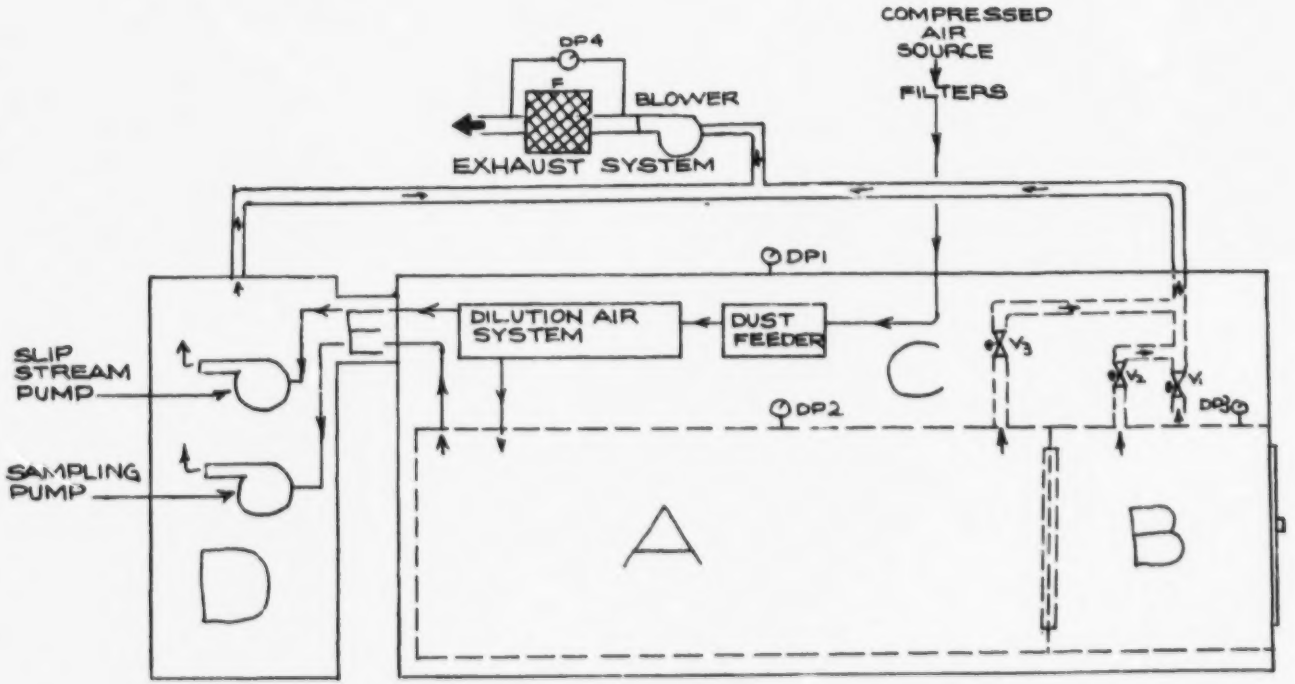


PLATE 1. INHALATION EXPOSURE FACILITIES FOR HAZARDOUS SUBSTANCES: EXPOSURE CHAMBERS FOR AIRBORNE TOXIC COMPOUNDS (A), AND FOR RADIO-LABELED BACTERIAL AEROSOLS (B AND C).



A Main exposure compartment
B Airlock for entry of animals and sampling filters
C Glove box – safety cabinet
D Accessory safety cabinet housing dry gas meter and pumps
E Duct connecting C and D
F HEPA filter

Differential pressure gauges:
DP_1 between room and safety cabinet C
DP_2 between compartments A and C
DP_3 between compartments B and C
DP_4 across HEPA filter

Valves controlling exhaust airflow from:
V_1 safety cabinet C
V_2 compartment B
V_3 compartment A

Figure 1. Schematic diagram of inhalation exposure chamber to toxic aerosols.

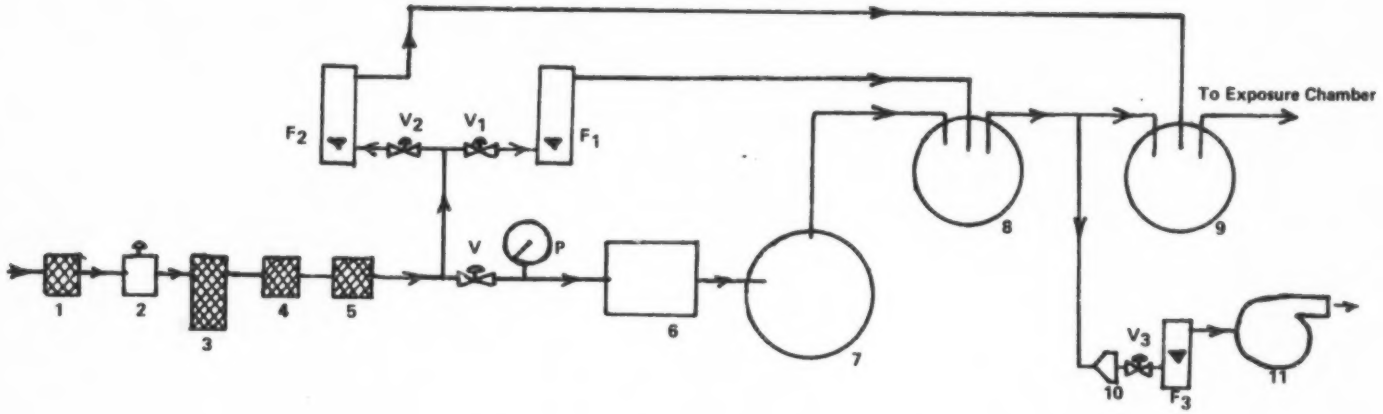
The sampling and slip stream pumps and a dry gas meter (measuring the air volumes through the Nuclepore sampling filters (Nuclepore Corp., Pleasantown, CA), used for monitoring aerosol mass concentration) were located in an adjacent glove box separated from the main safety cabinet to contain the heat generated by the pumps. The slip stream line leading to a collecting absolute filter, and the sampling line after the Nuclepore filter passed out of the main glove box through an air duct which connected to two safety cabinets.

Exhaust air flow from the exposure compartment, airlock and safety cabinet was controlled by three valves so that the differential pressures monitored by three Magnahelic gauges could be balanced as required. The pressure in the exposure compartment and airlock was maintained negative at all times relative to the safety cabinet even when maximum input air was added during aerosol generation or air purge following aerosolization.

All air exhausted from the exposure chamber, airlock and the safety cabinet was passed through a HEPA high efficiency particulate filter (Flanders Filters Inc., Washington, VA). To compensate for the pressure drop in the exhaust system caused by the resistance of the filter, a blower provided adequate air flow and negative pressure in the system during aerosol exposures and air purge. A differential pressure gauge measured the pressure drop across the HEPA filter and provided an indication when the filter was saturated. Exhaust from the chamber was shut off while the filter was changed.

Generation and Monitoring of Aerosols--The schematic diagram of the aerosol generation system used in the studies is shown in Figure 2. The compressed air supplied to the exposure chamber for dilution or dissemination of the test particles was passed through appropriate filter systems to dry the air and to remove all traces of oil and particulates. These included an Alemite coarse filter (Model 7620, Steward Warner, Chicago, IL) to dry the air, followed by a pressure regulator and a Deltech Model 130 oil filter (Deltech Engineering Inc., New Castle, DE). Finally before entering the chamber the air was further purified by passage through a MSA (Mine Safety Appliance Co., Pittsburgh, PA) absolute filter assembly to remove particles larger than 0.3 μm and a constant flow compressed air filter (Koby Inc., Marlboro, MA). A Wright dust feeder (L. Adams Ltd,, London, England) was used to generate aerosols of the copper smelter flue dust and the coal fly ash. The material to be aerosolized was compressed into a 13 mm diameter cylinder of the dust feeder. A slowly rotating blade continuously scraped off a small portion of the particles which were then entrained into a 21 liter/min air stream. The dust-laden air was impacted on a plate to break up the agglomerates and then passed through a settling chamber where the large particles were removed by sedimentation and after appropriate dilution the aerosol was introduced into the exposure chamber.

The purified air flow through the dust feeder was controlled by a valve and monitored by a pressure gauge. To obtain the desired mass concentration, the aerosol was passed through a two-stage dilution system consisting of two sequential dilution flasks controlled by two valves and monitored by two flowmeters. The primary system was capable of a three-fold dilution; for lower



1. Coarse filter
2. Pressure regulator
3. Deltech model 130 oil filter
4. MSA air line absolute filter
5. Koby air purifier and flow equalizer
6. Wright dust feeder
7. Settling chamber
8. Dilution flask #1
9. Dilution flask #2
10. Glass fiber filter holder
11. Vacuum/pressure pump

Valves controlling airflow:

V to dust feeder
V_1 to dilution flask #1
V_2 to dilution flask #2
V_3 through slip stream

Flowmeters for airflow:

F_1 to dilution flask #1
F_2 to dilution flask #2
F_3 through slip stream

P dust feeder pressure gauge

Figure 2. Schematic diagram of solid particulate aerosol generation system.

concentrations a secondary dilution was also used. In addition, any portion of the diluted aerosol could be removed by a vacuum/pressure-pump via a slip stream monitored by a flowmeter. Adequate airflow into the exposure chamber prevented drastic changes in temperature and humidity during aerosol exposures of animals in the chamber. The aerosol was introduced into the exposure chamber near the bottom at one end and exhausted along the top at the opposite end. A Rotron biscuit blower (Rotron Inc., Woodstock, NY) was operated continuously to further assure homogeneous distribution of the experimental environment.

Aerosol mass concentration in the chamber was monitored periodically during the exposure using a Model JM-7000 Aerosol, smoke and dust photometer (Phoenix Precision Instruments, Gardiner, NY) and a condensation nuclei counter (Type CN Small Particle Detector, Gardner Assoc., Inc., Schenectady, NY). These two instruments were used to assure that the aerosol concentration was in the required range during the entire exposure period, or if adjustments in the aerosol generation system were needed. Actual mass concentration was obtained in $\mu g/m^3$ from the gravimetric samples collected and the total volume of air sampled during the entire exposure period.

The photometer operates on the principle of forward light-scattering, detecting the intensity of light scattered by the suspended particulates sampled. This light intensity is proportional to the mass concentration of the aerosol. The photometer reading also depends on particle size, shape, refractive index and size heterodispersity and therefore the instrument has to be calibrated for the particular light-scattering qualities of each experimental powder. Readings on the condensation nuclei counter depend on the number of particles in the sampled aerosol. Both instruments are calibrated by gravimetric analysis of samples collected on membrane filters.

For the gravimetric determination samples of aerosols diluted to various mass concentrations were collected during the exposure period on 0.1 µm Nuclepore filters at constant flow rates using an oilless, teflon-lined pump (Model 907CA18-TFE, Thomas Industries, Inc., Sheboygan, WI). A dry gas meter (Model F-190 Rockwell International, MCU Div., Pittsburgh, PA) connected to the back side of the pump recorded the total volume of air sampled. Samples collected for sufficient time intervals, such that the weight of particles collected was 5 to 10% of the weight of the membrane filter, were weighed on a Mettler M30 electronic microbalance (Mettler Instrument Corp., Hightown, NJ) in the presence of a static eliminator. Air temperatures in the exposure chamber and at the dry gas meter were recorded so that the volume of air sampled and totalled by the gas meter could be corrected to chamber temperature at which the gravimetric sample was collected.

Aerosol particle size distribution was monitored periodically with an ASAS-300 PMT Active Scattering Aerosol Spectrometer (Particle Measuring Systems, Inc., Boulder, CO). Particles passing through the laser cavity of a continuous He-Ne laser produce pulses of light proportional only to their size and position in the beam. A pair of photomultiplier detectors image the light impulses and select pulses produced by particles in the correct sample space. A pulse height analyzer then determines the particle sizes.

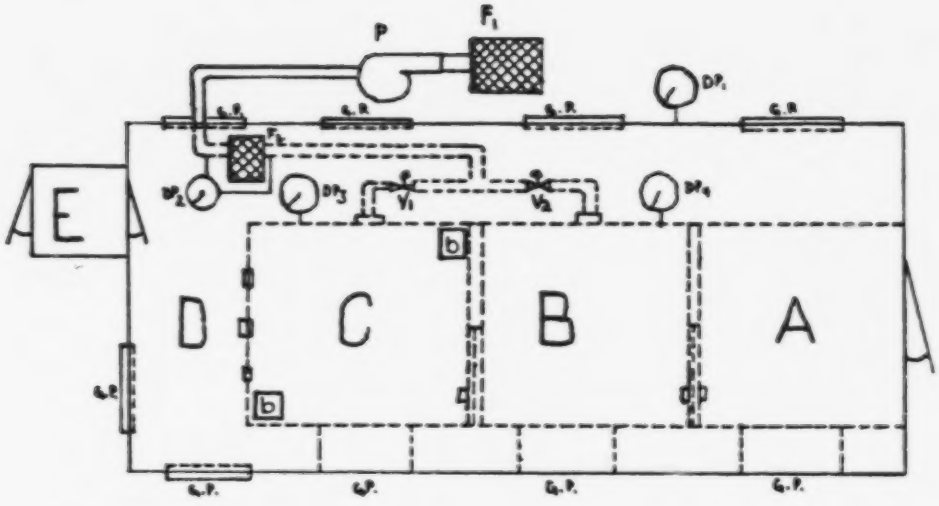
Exposure to Bacterial Aerosols--

Infectious aerosol exposure--Mice were challenged with bacterial aerosol in a 380-liter Plexiglas chamber installed within a microbiological safety cabinet. In this chamber, 144 mice housed in individual compartments of stainless steel wire cages, can be exposed to the aerosol simultaneously. Temperature and humidity in the aerosol chamber were maintained at 24 + 2°C and 65 + 5% RH. A continuous flow nebulizer (DeVilbiss Model 841, The DeVilbiss Co., Somerset, PA) was used to produce the bacterial aerosols with particles of <4 μm mass median diameter (MMD) as determined by a cascade impactor. Filtered air was supplied to the inlet of the nebulizer at a flow rate of 8 liters/min. Secondary air supply was maintained at 28 liters/min. The aerosol was sampled with an AGI-30 glass impinger (Ace Glass Inc., Vineland, NJ) containing 0.1% peptone as a collecting fluid. Upon completion of each bacterial aerosol exposure, ultra-violet germicidal lamps were used inside the safety cabinet and inside the exposure chamber, the exhaust air flow was increased, and the exposure chamber was purged with clean air.

Streptococcus pyogenes (Lancefield Group C) was used as the infectious challenge agent. To maintain the stock culture, mice were injected intraperitoneally with a suspension of *Streptococcus* and killed 24 hr later. Heart blood was incubated on blood agar for 48 hr at 37°C and *Streptococcus* colonies isolated from the agar were inoculated on blood agar with Todd Hewitt broth (THB) as the base. After 24-48 hr at 37°C, bacteria were harvested into Difco Nutrient broth (Difco Laboratories, Detroit, MI) with added 15% glycerol (v/v) and 1 ml aliquots were frozen at -70°C. For dissemination, 0.8 ml of the thawed bacteria was grown in THB at 37°C for 18 hr, harvested by centrifuging at 10,000 xg for 10 minutes, resuspended in 0.1% peptone water, and the concentration of bacteria adjusted to 10% transmittance in a Spectronic 20 spectrophotometer (Bausch & Lomb Inc., Northbrook, IL) at 440 mμ.

The inhaled dose of bacteria was determined by killing three mice immediately after the infectious challenge. The lungs were removed, weighed, and homogenized in 1.8 ml of sterile 0.1% peptone water. The suspension was diluted and plated onto blood agar and the colonies were counted after 48 hr incubation at 37°C. The inhaled dose expressed as number of viable bacteria per gram of lung tissue generally ranged, from 10 to 30 x 10^3 organisms.

Radioactive bacterial aerosol exposure--The chamber was installed in a glove box (Figure 3). To further prevent radioactive and pathogenic exposure hazard to laboratory personnel all air exhausted from the chamber system was passed first through an absolute filter (Model M. Watts Regulator Co., Lawrence, MA) placed within the glove box and subsequently through a HEPA filter located on the outside. The 87-liter main compartment of the chamber was accessible through appropriate airlocks to introduce or remove the animals and permit exposure of up to 72 mice held in individual compartments of wire cages. At the opposite end of the safety cabinet from the door through which the animal cages were passed, an additional small airlock provided a port for the nebulizer, impingers, as well as for the exchange of the exhaust line filters (Plate 1B and C). Two biscuit blowers controlled by a variac,



A,B	Airlocks for entry of animals
C	Exposure chamber
D	Safety cabinet
E	Airlock for entry of miscellaneous equipment
G.P.	Glove port
F_1	HEPA filter
F_2	Absolute filter
P	Blower (air pump)
b	Biscuit blowers
DP	Differential pressure gauges:
	DP_1 between room and safety cabinet, D
	DP_2 across absolute filter
	DP_3 between compartments C and D
	DP_4 between compartments B and D
V	Valves controlling:
	V_1 exhaust airflow from compartment C
	V_2 exhaust airflow from compartment B

Figure 3. Schematic diagram of radioactive bacterial aerosol exposure chamber.

assured homogeneous dispersion of the aerosol in the exposure chamber. The aerosol was produced by a Retec X-70 disposable nebulizer (Cavitron KDC, Inc., Anaheim, CA) using compressed air passed through appropriate filter systems. Airflow to the nebulizer was controlled by a flowmeter and passed through an electric solenoid valve, whereby aerosolization could be turned off using a timer. Secondary dilution air to the system provided adequate air-flow to maintain uniform temperature and humidity during the aerosol exposure (Figure 4).

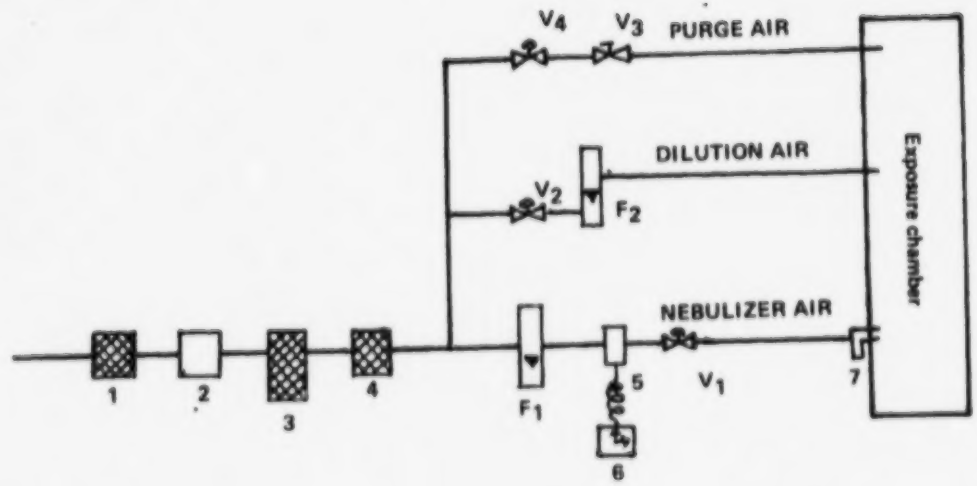
Before aerosolization total bacterial counts (BA) in a Petroff-Hausser microscopic counting chamber (Hausser Scientific, Blue Bell, PA) and radioactive counts (RA) by a liquid scintillation counter were immediately determined on the bacterial suspension to establish the concentration and radioactive labeling ratio (K = BA/RA). The radioactive labeling of the bacteria was adequate for monitoring if K <50. Based on the microscopic counts the nebulizer concentration was adjusted to 6×10^9 bacteria per ml. To obtain the required bacterial deposition in the lungs from the aerosol, approximately 15 ml of this bacterial suspension were aerosolized during a 30 min period with air flow rates of 6.5 liter/min through the nebulizer and 13 liter/min for secondary dilution air.

Cultures of ^{35}S-labeled *Klebsiella pneumoniae* were grown in Anderson's medium. The magnesium sulfate in the medium was replaced by magnesium chloride and the sulfate requirement of the bacteria was provided by addition of ^{35}S-labeled sodium sulfate. After harvest, the bacteria were washed and centrifuged at 12,000 xg 10 times for removal of unattached radiolabel. All bacterial counts were initially determined by dark field microscopy in a Petroff-Hausser bacterial counting chamber and subsequently also by culture plate technique.

Radioactive counts were measured with a Mark III Liquid Scintillation System Model 6880 (Searle Analytic Inc., Des Plaines, IL) using samples channels ratio to compute counting efficiency. The ^{35}S isotope adsorbs to the glass vials when liquid scintillation cocktails are used, resulting in up to 30% losses in total radioactive counts of the samples. To prevent this adsorption and to better suspend the lung homogenates, the samples for scintillation counting were prepared as gels, formed by adding 6.0 ml of Aquasol-2 (New England Nuclear, Boston, MA) and 3.0 ml of water to 1.0 ml aliquots of the lung homogenates or the bacterial suspensions.

Measurement of Experimental Parameters

Effects of 5, 10 or 20 daily 3-hr exposures to aerosols of the test particulates on mice were evaluated by observation of changes in their susceptibility to respiratory bacterial infection, pulmonary bactericical activity to inhaled radiolabeled *K. pneumoniae*, and by examination of the pulmonary free cells lavaged from their lungs. The effects were assayed within 1 hour after the last exposures to aerosols of the test particulates.



1. Coarse filter
2. Pressure regulator
3. Deltech model 130 oil filter
4. MSA air line absolute filter
5. Solenoid valve
6. Timer
7. Retec X-70 nebulizer

V Valves controlling:
- V_1 nebulizer air
- V_2 dilution air
- V_3, V_4 purge air

F Flowmeters monitoring:
- F_1 nebulizer air
- F_2 dilution air

Figure 4. Schematic diagram of radioactive bacterial aerosol generation system.

Infectivity--

In the experiments using the infectivity model system, animals exposed to the hazardous air pollutants and those exposed to filtered air were challenged simultaneously with the infectious agent so that mortality rates and survival time could be determined and compared within a given experiment. After the challenge, the mice were removed from the chamber and held in a clean-air, isolated animal room. During the 14-day observation period, deaths were recorded daily. The mean survival time was estimated according to the following equation:

$$MST = \frac{\Sigma(AxB)+(dxL)}{n}$$

where, A is the last day on which any individual mouse is alive, B is the number of mice surviving A days, d is the last day of observation, L is the number of mice alive on day d, and n is the number in the experimental group.

Pulmonary Bactericidal Activity--

Intrapulmonary bacterial inactivation was determined in the lungs of individual animals with a modification of the method of Green and Goldstein (13), whereby mice were exposed to radiolabeled live bacteria. The ratio of the viable bacterial count to the radioactive count in each animal's lung provided the rate at which bacteria were destroyed by the lung in a given time after infection. After exposure to the test compounds, groups of mice were infected by exposure to aerosols of ^{35}S-labeled *K. pneumoniae*. Immediately (0 hr) and at 3 hr after the infectious challenge, 5 (0 hr) and 12 (3 hr) mice, respectively, from each treatment group were killed and their lungs were aseptically removed and homogenized. From the radioactive counts made on aliquots of the homogenate and the radioactive labeling ratio determined for each bacterial culture in 0-hr lung samples, the total number of bacteria deposited in each lung was calculated. On the basis of this information another aliquot of each lung homogenate was appropriately diluted to determine by plate count technique the viable bacteria that remained in the lungs.

Alveolar Macrophages and Other Pulmonary Free Cells--

Alveolar macrophages were obtained from the mice by tracheobronchial lavage. Mice were killed by intraperitoneal injection of sodium pentobarbital and the lung-heart-trachea complex was removed from the chest cavity *in toto*. A blunted hypodermic needle inserted into the trachea and fastened with surgical suture was used to lavage the lungs by repeated infusions of warm (37°C) saline. After the cells were isolated from the lavage fluids by centrifuging at 365 xg and washing in HBSS, viability, total and differential cell counts, and cellular ATP levels were determined as described earlier in the section for *in vitro* methodology. ATP levels were expressed as femtograms of ATP per 10^5 AM.

Data Analysis

Results of the experiments were subjected to statistical analyses and the significance of the observed differences were reported at the 10% probability level. As appropriate, Chi-square test and Student's t test were used.

SECTION 5

RESULTS AND DISCUSSION

TEST PARTICLES

The objective of these studies was to determine if *in vitro* exposure of alveolar macrophages to a series of industrial particles resulted in the same relative toxicity ranking as *in vivo* exposure of the intact animal on the host defense system. To conduct such a comparative *in vitro* and *in vivo* evaluation required: (1) complex industrial particles, (2) in sufficient quantities for aerosol exposures in the *in vivo* studies, and (3) ranging in toxicity from relatively non-toxic to highly toxic so that particles of differing toxicity could be selected for the *in vivo* studies. As a result of the large sample requirement, it was not possible for the IERL at that time, to provide sufficient quantities of true emission samples collected by use of special emission source samplers located after the normal in-plant control devices. It was therefore, decided to utilize particulate samples obtained directly from the in-plant control devices whether they be baghouses, ESP's, or cyclones. None of the samples reported here are emission or effluent samples collected after the pollution control devices and are not necessarily similar in composition or toxicity to emission samples. Samples obtained from the control devices such as those used in these studies are generally removed from the effluent stream at very high temperatures prior to cooling and dilution. The more volatile compounds, which may also be more toxic, are not collected and, therefore, are not present on these particles. Thus the health effects data reported in these studies do not reflect the true effects of the actual fugitive emissions from the various stationary point sources described.

IN VITRO STUDIES

The original experimental design called for an *in vitro* screening of all the test particles with rabbit AM to be followed by further *in vitro* evaluation of selected samples with mouse AM before starting the *in vivo* exposures in mice. This approach was planned in order to bridge the species change from rabbits to mice between *in vitro* and *in vivo* exposures. Rabbits are the established choice for *in vitro* experiments because of the high yields of AM that can be obtained from their lungs, whereas mice are selected for the *in vivo* studies because a considerable amount of background information is available on them pertaining to susceptibility to both infectious diseases and air pollutants. Moreover, large numbers of mice can be utilized to obtain sufficient replications of experiments, thus enabling statistical analyses of results. However, an exploratory study showed that AM lavaged from normal

mice did not maintain viability during *in vitro* incubation as well as those obtained from rabbits. Rabbit AM viability in control cultures after 20 hr incubation was routinely found unchanged from the viability values obtained immediately after lavage (96-98%), whereas in the case of AM obtained from mice viability was considerably lower after *in vitro* incubation than at isolation.

To evaluate the toxic ranking of the same test particles with the AM cytotoxicity assay using cells from either species and to correlate the data, experimental conditions had to be established under which control cells maintained *in vitro* would respond similarly whether obtained from rabbits or from mice. We therefore examined changes in experimental variables that would lead to an improved maintenance of viability in mouse AM in *in vitro* cultures. In the course of these studies we found that viability was not affected by the use of various media, or various lots or sera, or the temperature of the lavaging media (37° or 0°C), nor did we discover any differences in viability related to mouse strain, age or sex. (Use of very young mice was found disadvantageous because of the low cell counts frequently encountered in their lung lavages.) Our experiments demonstrated that *in vitro* viability in AM of mice could be maintained after 20 hr in control cells unchanged from the values obtained immediately after lavage only if the incubation medium contained 50% instead of the 10% heat-inactivated calf serum used for rabbit AM.

In an effort to examine the effect of animal variability on *in vitro* AM viability cells lavaged from the lungs of ten mice were isolated separately for each animal and subsequently incubated in individual and in pooled samples for 20 hr at 37°C using 50% serum supplement in the medium. Comparison of the viability values determined at isolation and after the 20 hr incubation period showed that although viabilities at isolation ranged between 93.5 and 96.5%, after incubation the AM of three out of ten mice were lower than 90%, namely 85.0, 88.5 and 79%. When the AM obtained from the first five, the second five or from all ten mice were pooled the viabilities determined were: 89.5, 95 and 93% respectively. These observations indicated that there was a certain risk in pooling cells for the cytotoxicity assay. Even if we would initially check AM viability in every individual lavage and *a priori* discard those with low viability, we could not be assured of uniform response in the cell pool. On the other hand, because of the low yields in AM from mice, using individual mice for each exposure point not only would have increased the complexity and time requirement of the procedures, but would have prevented the design of a meaningful dose response experiment that included proper internal controls. Thus it was decided not to pursue further the planned *in vitro* studies with mouse AM.

The *in vitro* effects of the various particulate samples incubated for 20 hr at 37°C with rabbit AM were monitored in dose response experiments. Cell viability, total protein and ATP levels served as experimental parameters. The means and standard errors shown in Figure 5 were calculated from six or nine replicate observations at each concentration with triplicate assay determinations at each point. Viability

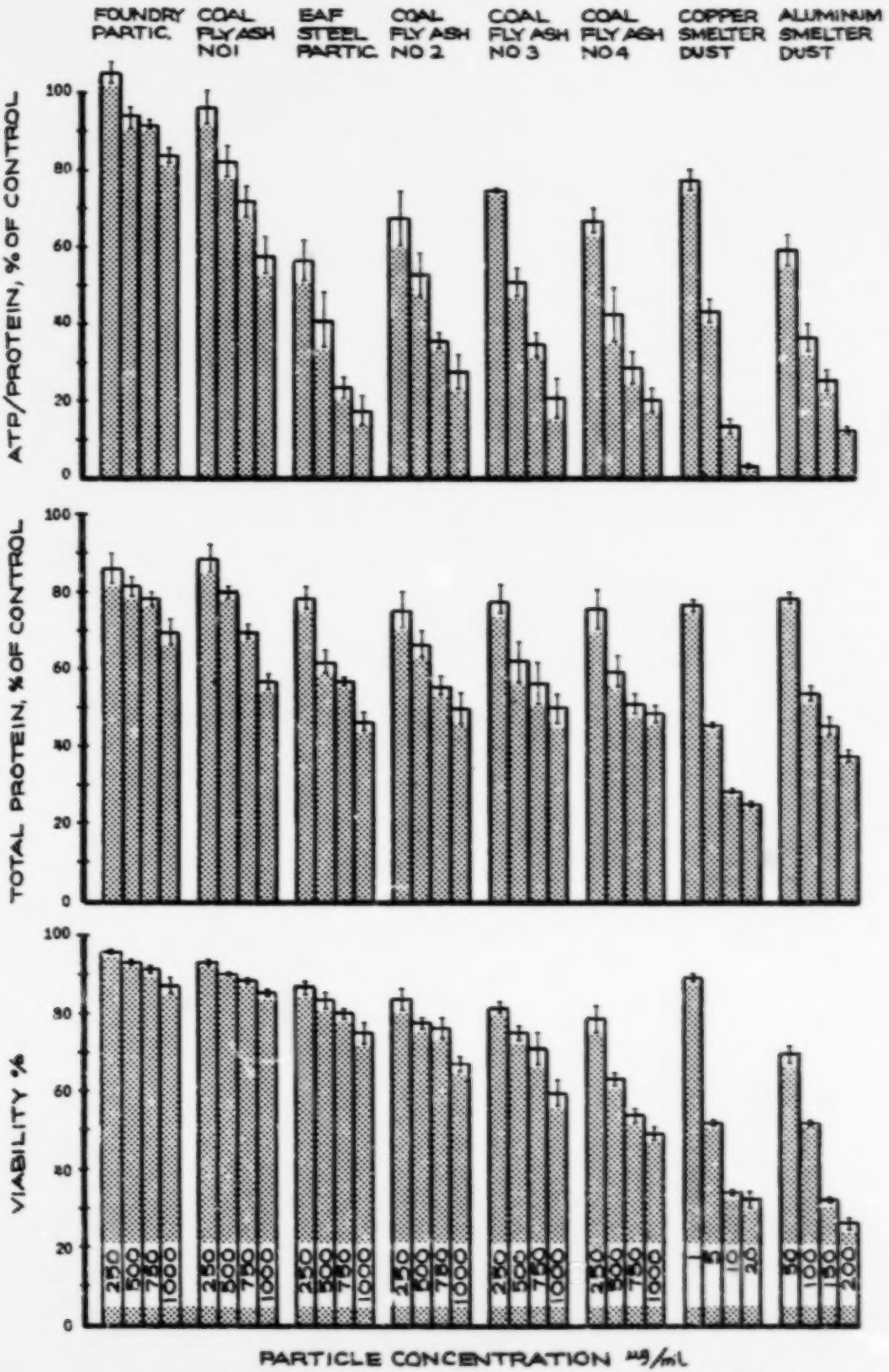


Figure 5. Effect of *in vitro* particle exposure on viability, total cellular protein and ATP levels in rabbit alveolar macrophages. Means ± SE of six to nine replicate observations are shown.

is shown as a direct percentage of the number of cells in the sample, and total protein and ATP per protein are expressed as percent of the control responses. Values for zero exposure concentration are not shown, since AM viability ranging generally from 96 to 99% was unchanged by the incubation period, and for the other parameters the values for zero exposure concentration at 20 hr were by definition 100%.

Regression analysis applied to the data showed a significant negative linear dose response relationship for each parameter in all samples (p ranging from 0.01 to 0.001). For particles collected from a steel foundry, an electric arc furnace steel plant, and from four coal-fired power plants (Fly Ash Nos. 1, 2, 3 and 4) a stepwise decrease for all three parameters could be observed with increasing particle concentration in the range of 250 to 1000 µg/ml. All these changes demonstrated highly significant linear dose responses.

The samples collected from a copper and an aluminum smelter had somewhat different effects. Within the concentration range of 250 to 1000 µg/ml these two dusts produced great decreases in viability relative to controls that became more pronounced for total cellular protein, and were in some cases almost too low to detect for ATP levels. These changes did not appear to be dose related in the same fashion as for the rest of the samples. In order to obtain similar monotonicly decreasing responses for all three parameters to those observed with the other particles the exposure concentration range had to be lowered to between 1 and 20 µg/ml for the copper, and to 50 and 200 µg/ml for the aluminum smelter samples. Undoubtedly, a larger experimental error was introduced into sample preparation when solid particles were weighed and their suspensions diluted in this extremely low concentration range than when using particulate suspensions at higher concentrations. Nevertheless statistically significant negative linear dose response relationships were obtained for both samples in these low concentration ranges, although not at the higher concentrations tested.

The lines estimated by linear regression analysis for viability from the experimentally determined values are plotted in Figure 6 to demonstrate the different effects on AM.

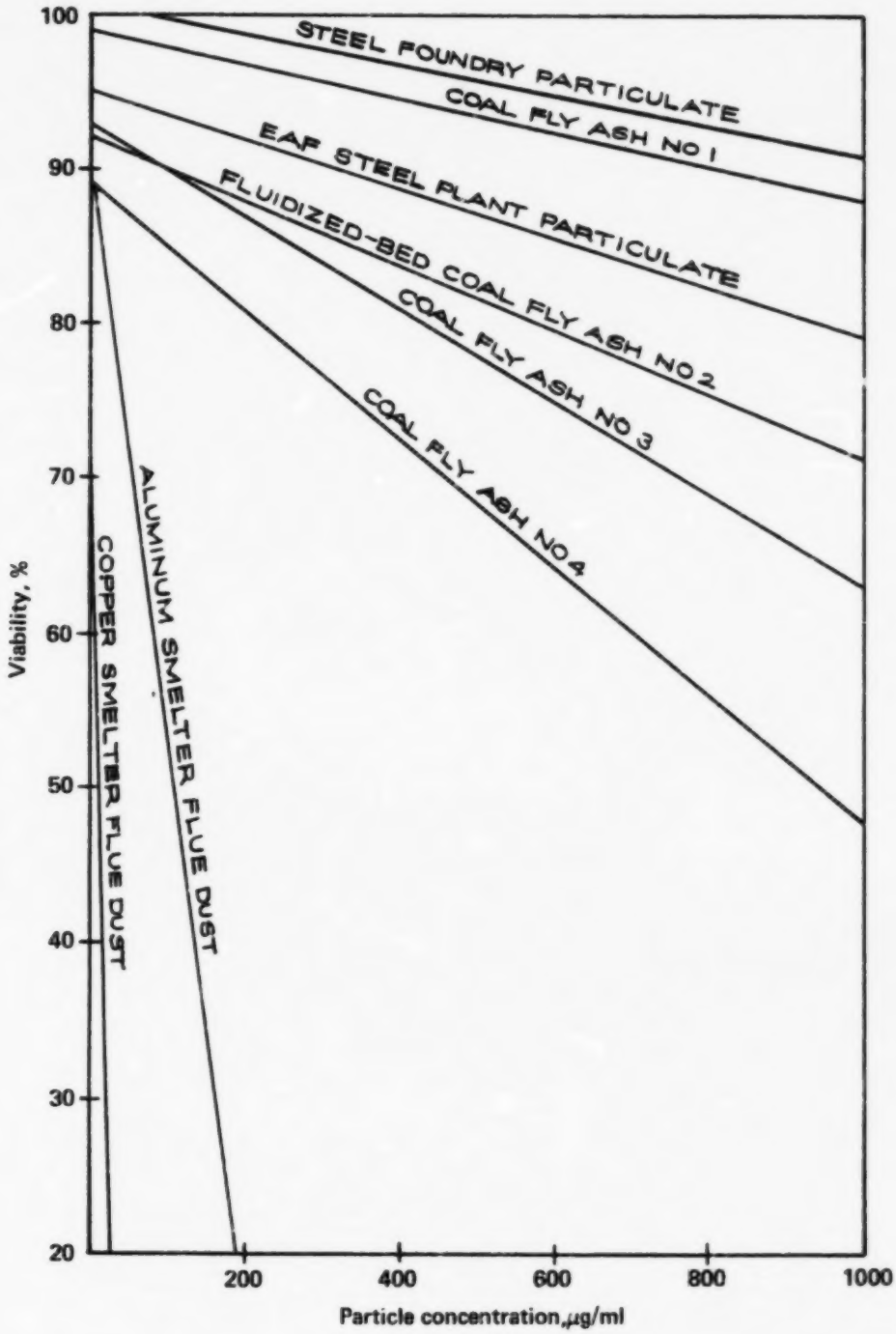


Figure 6. Effect of *in vitro* particle exposure on rabbit alveolar macrophage viability. The statistically estimated linear regression lines were determined from six to nine replicate observations.

The estimated concentrations required to reduce the experimental parameters to 50% of the control responses were calculated from the linear regression lines and based on these data a relative toxic ranking of the various particulate pollutants was established. The samples collected from the copper and aluminum smelters were most cytotoxic with the copper smelter particles being the more toxic of the two (Table 1). Power plant fly ash No. 4 showed intermediate cytotoxicity, whereas the EAF steel plant particles and coal fly ash Nos. 3 and 2 showed moderate to low cytotoxicity. The remaining two samples had only slight effect on AM, so that the EC_{50} could be obtained for all parameters only by extrapolation above the actual highest concentration used; for fly ash Nos. 2 and 3, and the EAF steel plant sample this was only true for viability EC_{50} estimation.

TABLE 1. CONCENTRATION[a] OF PARTICLES REQUIRED TO REDUCE ALVEOLAR MACROPHAGE VIABILITY, TOTAL PROTEIN CONTENT AND ATP LEVELS TO 50% (EC_{50})

	EC_{50} (µg/ml)		
Sample	Viability	Total Protein	ATP/Protein
Steel Foundry Particle	>1000[b]	>1000[b]	>1000[b]
Coal Fly Ash No. 1 (Conventional)	>1000[b]	>1000[b]	>1000[b]
Coal Fly Ash No. 2 (Fluidized-Bed)	>1000[b]	952	537
Coal Fly Ash No. 3 (Conventional)	>1000[b]	930	553
EAF Steel Plant Particle	>1000[b]	895	332
Coal Fly Ash No. 4 (Conventional)	949	856	445
Aluminum Smelter Dust	114	139	60
Copper Smelter Dust	11	6	5

[a] Estimated by linear regression analysis for experimental parameters expressed as: Viability %; Total Protein (µg), % of Control; ATP/Protein (fg/µg), % of Control.

[b] Highest concentration tested.

In classical statistical terms it is not permissible to make estimates from extrapolated values. Thus, to obtain the relative toxicity for these samples without extrapolation, the values for the various parameters were calculated from the linear regression equations for the median of the particle concentration ranges used. (The equation was solved for y instead of x). Table 2 shows a toxic ranking at 500 µg/ml for all samples that had significant regressions in the experimental dose range of 0 to 1000 µg/ml. The data demonstrate that among these samples coal fly ash No. 4 was the most toxic with all other samples having lesser cytotoxicity among the three for which EC_{50} values could be estimated without extrapolation. Thus by using the coal fly ash No. 4 in both statistical estimates, we could in fact link the toxic ranking for all particulate samples tested.

TABLE 2. ESTIMATED EFFECT ON VARIOUS EXPERIMENTAL PARAMETERS IN MACROPHAGES AFTER INCUBATION WITH THE PARTICLES AT THE MEDIAN OF THE EXPOSURE CONCENTRATION RANGE[a]

Sample	Viability	Total Protein	ATP/Protein
Steel Foundry Particulate	96.0	81.4	96.9
Coal Fly Ash No. 1 (Conventional)	93.9	78.8	83.1
EAF Steel Plant Particulate	87.2	65.8	40.9
Coal Fly Ash No. 2 (Fluidized-Bed)	81.7	65.8	52.1
Coal Fly Ash No. 3 (Conventional)	77.7	65.5	53.8
Coal Fly Ash No. 4 (Conventional)	68.4	62.8	46.2

[a] Estimated for the median exposure concentration of 500 µg/ml by linear regression analysis for experimental parameters expressed as: Viability %; Total Protein (µg), % of Control; ATP/Protein (fg/µg), % of Control.

It can also be seen from both tables that cell viability appears to be less affected by exposures to any of the particulate matter than total protein or ATP levels. Nevertheless, the decreases in total protein levels, representing the extent of cell lysis, and in cellular ATP levels, indicating impairment in phagocytic capacity, generally paralleled those observed for viability.

The dye exclusion-viability assay used in the studies determines the percentage of intact viable cells remaining at the end of the exposure period and does not take into account the number of cells destroyed by lysis. Yet viability and cell lysis may be affected to different extents by the same test agent and thus their combined effect is an important index of overall cell damage. To assess this effect Waters *et al.* (5) investigating the cytotoxicity of soluble metal salts on AM have introduced the "viability index". To estimate the index value the determined cell viability is multiplied by the ratio of intact cells remaining in the test culture to the number of cells remaining in the control culture. We found cell counts in a hemocytometer after exposure to and in presence of solid particles to be erratic and therefore unreliable for monitoring cell lysis. Total protein levels on the other hand gave us a good measure of cell lysis and cell fragility (the centrifugation and washing process for elimination of the serum supplement also removes some of the marginally damaged cells). The ratio of total cellular protein levels in the test over control cultures was used to obtain the correction factor for the viability index.

$$\text{Viability Index} = \text{Viability \%} \frac{\text{Total protein in test culture}}{\text{Total protein in control culture}}$$

By multiplying the means of percent viability with the means of the total protein in test over control cultures at each concentration tested we found significant (p ranging from 0.05 to 0.1) negative linear dose response relationship for the viability index in the concentration range of 250 to 1000 µg/ml for all but the aluminum smelter samples. For the latter a significant correlation could be obtained in the range of 50 to 200 µg/ml. The EC_{50} values estimated for the viability index and summarized in Table 3 show that only for the steel foundry particles does the EC_{50} concentration exceed the experimental concentration range, whereas for the uncorrected viability this was true for five samples, as could be seen earlier in Table 1. No EC_{50} value was calculated for the copper smelter dust because the viability index remained under 20% within the exposure range of 250 to 1000 µg/ml. (For the 1 to 20 µg/ml range the viability index did not show significant dose response correlation). Thus a toxic ranking paralleling those previously shown was established. The copper smelter dust again showed the highest cytotoxicity with particles from the aluminum smelter following closely. Coal fly ash Nos. 4 and 3 showed intermediate, fly ash No. 2 and the EAF steel plant particles moderate, and fly ash No. 1 and the steel foundry particles lowest toxic effect.

TABLE 3. CONCENTRATION OF PARTICLES REQUIRED TO REDUCE ALVEOLAR MACROPHAGE VIABILITY INDEX TO 50% (EC_{50})[a]

Sample	EC_{50} (µg/ml)
Steel Foundry Particle	>1000
Coal Fly Ash No. 1 (Conventional)	979
EAF Steel Plant Particle	617
Coal Fly Ash No. 2 (Fluidized-Bed)	555
Coal Fly Ash No. 3 (Conventional)	494
Coal Fly Ash No. 4 (Conventional)	343
Aluminum Smelter Dust	44
Copper Smelter Dust	--[b]

[a] Estimated by linear regression analysis. For details and definition of viability index see text.

[b] The viability index was under 50% throughout the tested concentration range.

The relatively high cytotoxicity of the copper and aluminum smelter dust samples at the low concentrations and the absence of a dose-response at higher concentrations suggested that soluble compounds released into the medium during the incubation were responsible, in addition to the particles *per se*. To substantiate this hypothesis the test particles without AM were preincubated in the medium at the highest exposure concentration (1000 µg/ml) and under similar conditions (20 hr, 37°C) to those used in the cytotoxicity experiments. When AM were subsequently incubated for 20 hr at 37°C with the original particle suspension, the resuspended particles, and portions of the filtered culture media, viability decreased equally after exposure to the

preincubated original particle suspension, or the preincubated particles resuspended in fresh maintenance medium. The ultracentrifuged and filtered suspension media did not produce decreases in AM viability relative to the unexposed control AM for all samples with the exception of the aluminum and the copper smelter dusts. This clearly demonstrated that, if any, the amounts solubilized from coal fly ash, steel foundry or EAF steel plant particles were not toxic to the AM. However, as evident from the data, soluble cytotoxic components were released during incubation from copper and aluminum smelter samples and the preincubated particles remained cytotoxic as well (Table 4). In subsequent tests at <1000 µg/ml (not shown in this table) it was also found that supernatants of suspensions of the two smelter samples decreased AM viability at all concentrations examined.

TABLE 4. EXAMINATION OF TEST PARTICLES FOR SOLUBLE CYTOTOXIC COMPONENTS BY ALVEOLAR MACROPHAGE VIABILITY

Sample	AM-Viability %[a]		
	P+S	P	S
Steel Foundry Particle	81.9	85.3	97.5
EAF Steel Plant Particle	84.2	81.5	96.7
Coal Fly Ash No. 1 (Conventional)	74.3	70.1	98.3
Coal Fly Ash No. 2 (Fluidized-Bed)	83.3	80.3	97.3
Coal Fly Ash No. 3 (Conventional)	75.2	76.5	96.7
Coal Fly Ash No. 4 (Conventional)	65.7	64.3	97.0
Aluminum Smelter Dust	29.8	31.8	46.8
Copper Smelter Dust	56.0	53.5	65.1

[a] Determined after exposure at 37°C for 20 hr at concentrations of 1000 µg/ml to the following fractions of the preincubated particles:

P+S Original particle suspension
P Particles separated from medium
S Particle-free medium

All control viabilities ranged from 96 to 98%.

Thus the mechanism responsible for the cytotoxic effects for the two dusts appears to be more complex than for the other insoluble particles; in addition to the effect of the particles themselves it depends on the nature of the soluble toxic components and the rate of their release into the medium.

Spark-source mass spectroscopic analysis of the copper smelter particles conducted by EPA's IERL showed that major (>1%) trace metal constituents with concentrations ranging up to 20% by weight were Pb (18%), As (13%), Cu (8%), Fe (3%), Sb (3%), and Zn (2%). Subsequent spark-source mass spectroscopy of the ultracentrifuged and filtered supernatant fraction of the copper smelter particles showed solubilized arsenic being present at a concentration that was by three orders of magnitude higher than the background concentration in the original incubation medium (i.e., 459 versus 0.1 µg/ml). A slight increase in the concentrations of cadmium and antimony in the supernatant (from 0.4 to 9.4 and 0.5 to 9.1 µg/ml respectively) could also be discovered, but there was no evidence for the solubilization of any other trace metals. The presence of solubilized arsenic in the supernatant was also confirmed by ion chromatographic analysis. Thus the evidence from these analytical data suggests that water-soluble arsenic may indeed by a major component responsible for the cytotoxicity of the copper smelter particles. At the present there is no information available on the soluble components in the aluminum smelter dust.

The *in vitro* Rabbit Alveolar Macrophage (RAM) test has enabled us to evaluate the relative cytotoxicity of eight industrial particulate effluent samples. The data have demonstrated that particulates from a steel foundry and an electric arc furnace steel plant and fly ash samples from three conventional combustion processes and one fluidized-bed process, had low, moderate to intermediate effects on AM. The samples collected from a copper and an aluminum smelter ranked extremely high in cytotoxicity relative to the others, with the copper smelter sample being the most toxic. In case of the two smelter samples in addition to the particles *per se* soluble components released during incubation were contributing to the total cytotoxic effect.

IN VIVO STUDIES

A major objective of a phase of these studies was to confirm the relevance of the *in vitro* assay by demonstrating parallel *in vivo* effects upon inhalation of these compounds. Mice were exposed to aerosols of the copper smelter dust and the fluidized-bed coal fly ash (No. 2), i.e., particles that showed very high and low cytotoxicity *in vitro*.

Aerosol concentrations were 2 and 1 mg/m^3 for the coal fly ash and 2, 1 and 0.5 mg/m^3 for the copper smelter dust. The aerosol mass concentrations were determined by taking the averages of three daily filter readings and calculating the means and standard deviations (SD) over the total number of exposure days. The means and SD for the copper smelter dust were

2050 ± 190 μg/m^3, 1020 ± 180 μg/m^3 and 540 ± 100 μg/m^3. For the fluidized-bed coal fly ash the concentrations were 2040 ± 370 μg/m^3 and 1010 ± 160 μg/m^3. Both aerosols had log-normal size distribution with count median diameter and σ_g-s of 0.225 μm and 2.9 for the copper smelter dust and 0.134 μm and 2.1 for the fly ash, respectively.

The effects were determined after 5, 10 and 20 exposures by examining changes in the composition of pulmonary cellular lavage, susceptibility to respiratory streptococcal infection, and in the bactericidal activity to inhaled radiolabeled *K. pneumoniae* in lungs of mice. Results are summarized in Table 5 and are expressed for the pulmonary free cell data as percentage of the control responses, and for mortality rates (%), mean survival times (days) and pulmonary bactericidal activity (%) as the change between exposed and control observations.

Inhalation of the aerosols generally did not affect the free cells lavaged from the lungs in a manner that could be attributed to the multiple exposures. There was a greater number of significant changes in total cell counts and ATP levels (Figure 7) in the copper smelter dust-exposed mice than in the fly ash-exposed mice, although these changes were not consistent and could not be related to the various exposure conditions. Furthermore, differential cell counts and percent viability of the lavaged cells (not shown) were not affected by the exposures.

Cnanges in susceptibility to streptococcal infection could clearly be correlated with inhalation of the particulate samples. Figure 8 shows the difference in percentage mortality due to streptococcal pneumonia between the aerosol-exposed and filtered air-exposed control mice. The mortality due to streptococcal infection increased significantly after 5, 10 and 20 daily 3-hr exposures to aerosols of 2050 μg/m^3 copper smelter dust. No significant changes were found at 1020 μg/m^3 with the same multiple exposure schedule, nor at 540 μg/m^3 after 5 exposures. However significant increases in mortality were found after 10 and 20 daily 3-hr exposures to 540 μg/m^3 copper smelter flue dust. Thus the no-measurable effect in this case was definitely below 2050 μg/m^3 aerosol concentration and the data also indicate that with more replicate studies using additional mice a lower no-effect concentration could have been established at one of the other concentration levels tested.

Inhalation of 2040 and 1010 μg/m^3 of the fluidized-bed coal fly ash did not affect susceptibility to streptococcus infection after any of the multiple exposure conditions. Mean survival times (Table 5) showed significant decreases paralleling the significant increases in mortality rates, except after 20 exposures to 540 μg/m^3 copper smelter dust.

The bactericidal activity to inhaled radiolabeled *K. pneumoniae* (Figure 9) decreased significantly after 5, 10 or 20 daily 3-hr exposures to 2050 and 1020 μg/m^3 copper smelter dust aerosol. Upon exposure to 540 μg/m^3 a significant decrease was seen after 20 but not after 5 or 10 exposures. Thus in terms of pulmonary bactericidal activity the no-measurable effect level for the copper smelter dust was <1 mg/m^3 and appeared to be closer to 0.5 mg/m^3.

TABLE 5. EFFECTS OF MULTIPLE DAILY 3-HR EXPOSURES TO AEROSOLS OF COPPER SMELTER DUST OR COAL FLY ASH NO. 2 ON THE PULMONARY FREE CELLS, ON SUSCEPTIBILITY TO RESPIRATORY STREPTOCOCCUS INFECTION AND ON BACTERICIDAL ACTIVITY TO INHALED ^{35}S-*K. PNEUMONIAE* IN CD$_1$ FEMALE MICE

Aerosol Exposure				% of Control				% Change: Exposed-Control				
				Total Cells		Cellular ATP		Streptococcus Inf.			Bactericidal Activity	
Sample	Conc, μg/m^3 Mean	SD	No. of Exposures, 3 hr/day	n[a]	Count x 10^9	n[a]	10^{-8}g/10^9 cells	n[a]	Mortality %	MST days	n[a]	% *K. pneumoniae* killed
Copper Smelter Dust	2060	190	5	39	77*	31	100	166	33.6***	-2.5***	55	-23.7***
			10	24	94	17	93	216	27.2***	-2.9***	46	-17.0***
			20	16	107	16	126*	268	19.0***	-1.3**	45	-12.8***
	1020	180	5	34	78*	33	118	280	1.4	0	45	- 7.0**
			10	33	88	31	86	233	6.3	-0.2	42	- 9.7***
			20	20	59**	16	96	241	-3.2	0.3	74	- 4.1**
	540	100	5	20	120	17	88	286	-1.4	0.1	75	- 2.8
			10	17	103	15	79**	195	6.4*	-0.6**	64	4.1
			20	19	107	17	90	308	11.5**	-0.4	45	- 5.4***
Coal Fly Ash No. 2 (Fluidized-bed)	2040	370	5	33	106	31	106	325	0.1	-0.2	60	2.2
			10	21	85	19	102	276	-3.4	0.1	68	2.5
			20	38	94	33	89**	299	1.6	-0.1	69	0.8
	1010	160	5	34	86	31	101	280	-4.3	0.5	42	3.5**
			10	37	88	33	87	205	-0.6	0.2	44	3.7***
			20	19	100	16	98	192	-2.9	0.1	43	- 2.8

[a] number of mice

Significant change from corresponding control mice: *$p \leq 0.1$ **$p \leq 0.05$ ***$p \leq 0.001$

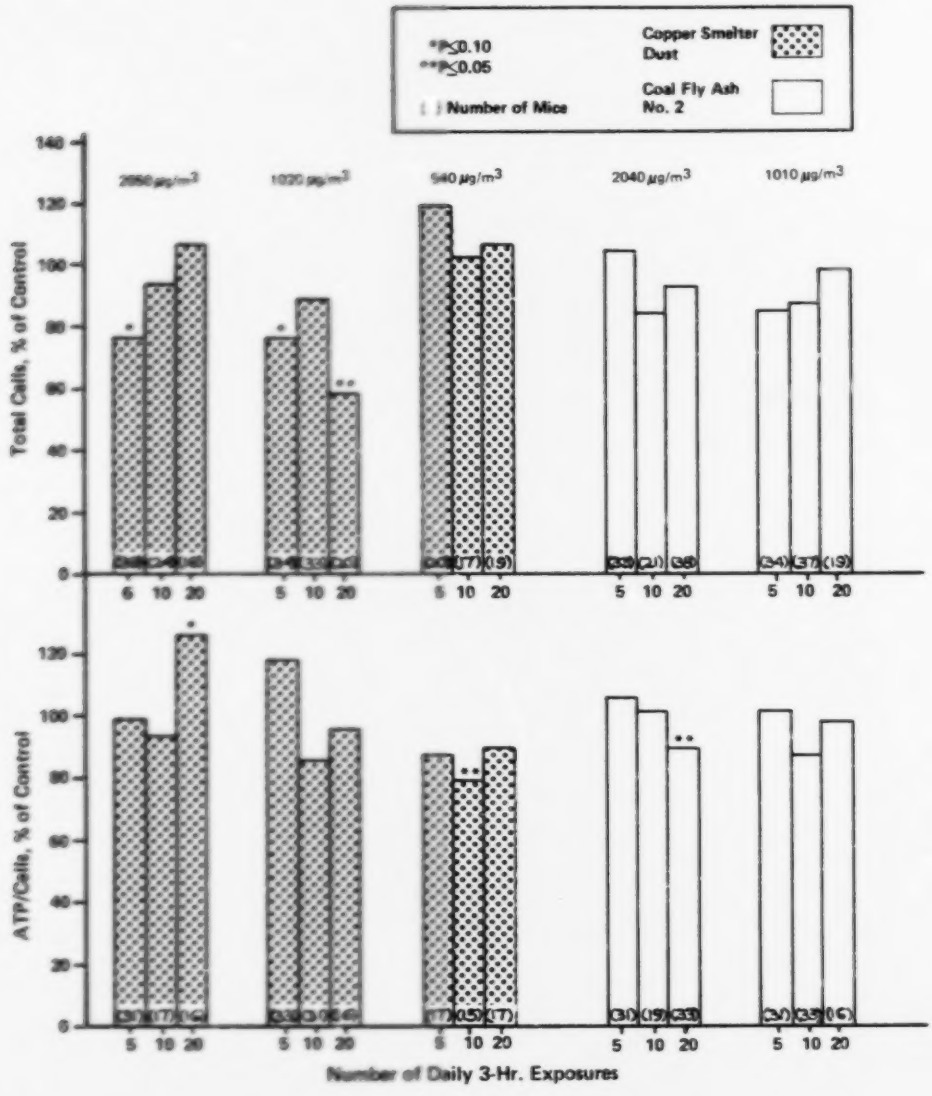


Figure 7. Effects of multiple 3-hr daily exposures to aerosols of Copper Smelter Dust or Coal Fly Ash No. 2 on total cell counts and cellular ATP levels determined in the pulmonary cellular lavage of mice.

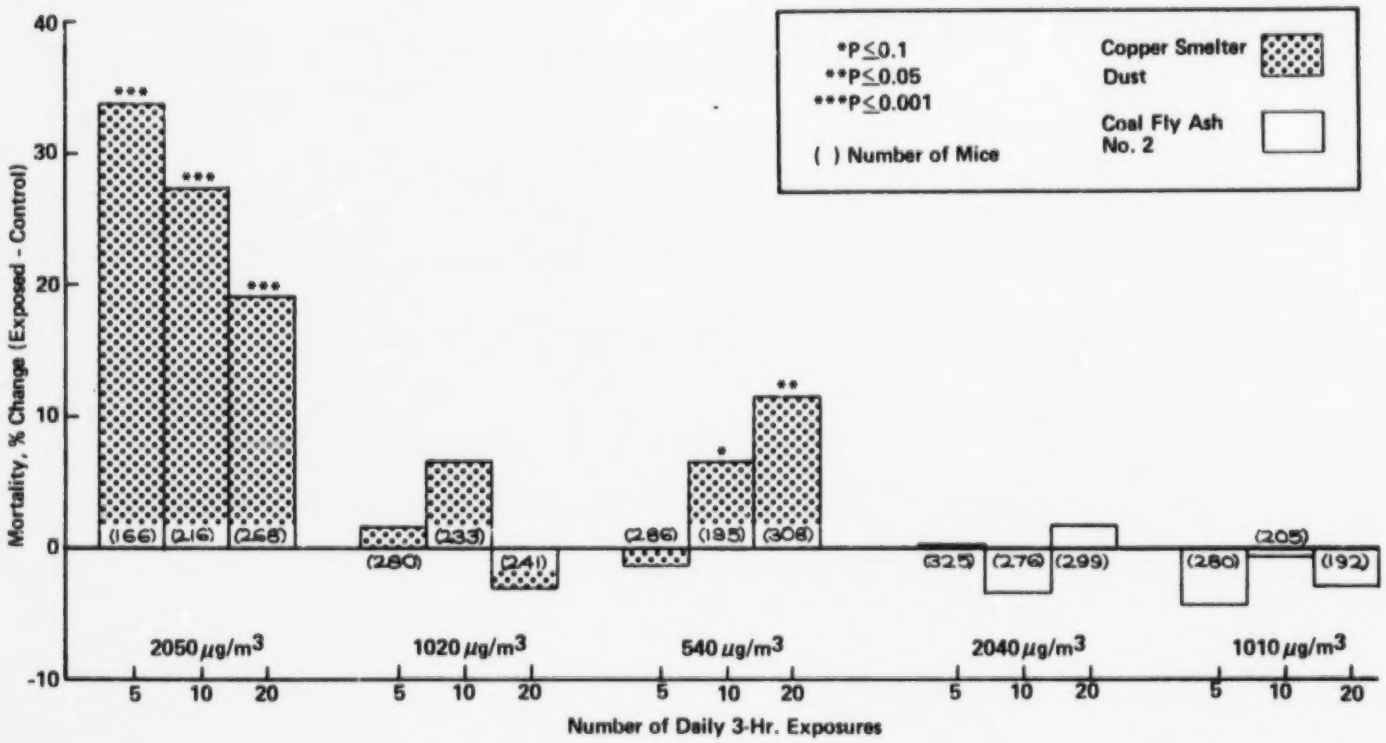


Figure 8. Changes in mortality in mice from streptococcal pneumonia after multiple 3-hr daily exposures to aerosols of Copper Smelter Dust or Coal Fly Ash No. 2.

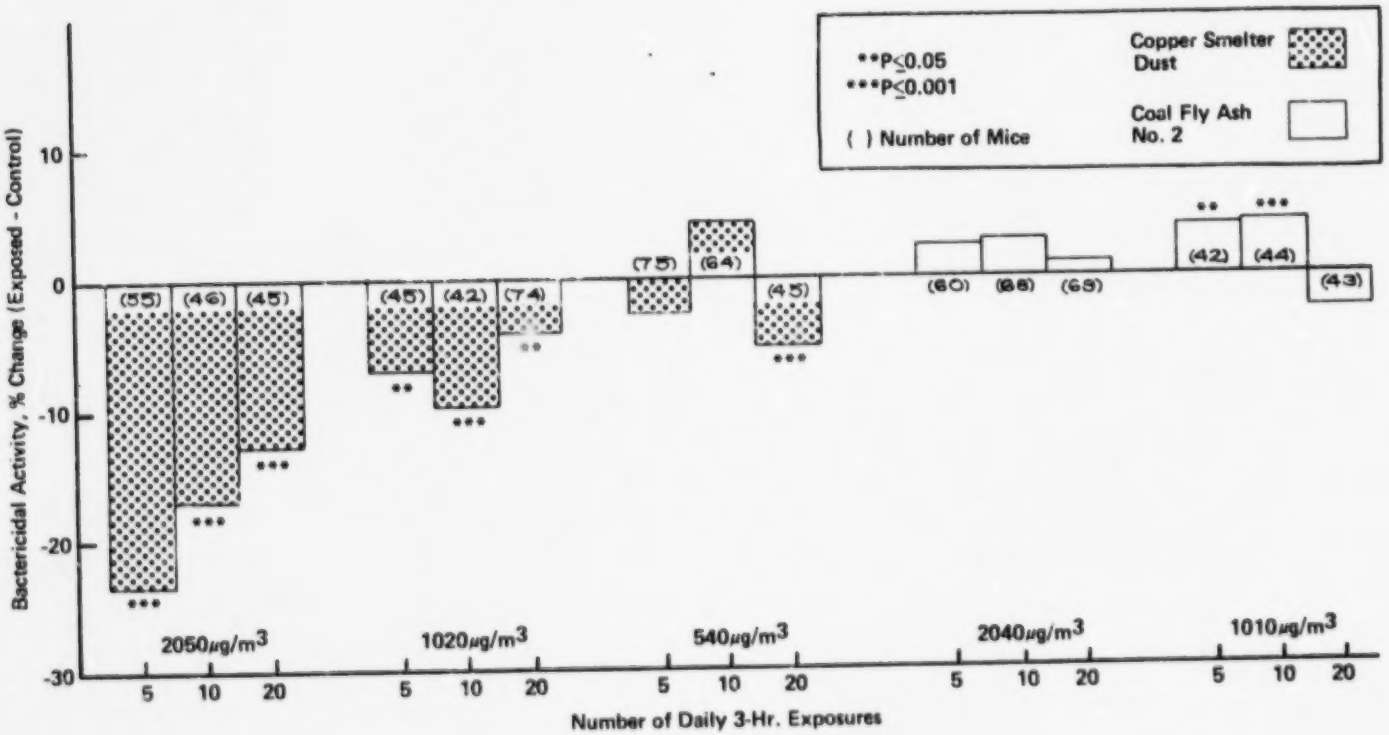


Figure 9. Changes in pulmonary bactericidal activity in mice to inhaled ^{35}S - *K. pneumoniae* after multiple 3-hr daily exposures to aerosols of Copper Smelter Dust or Coal Fly Ash No. 2.

Mice exposed to the fluidized-bed coal fly ash generally showed increases in bactericidal activity when compared to the filtered air-exposed controls. The increases were not significant after multiple exposures to 2040 $\mu g/m^3$, but were significant after 5 and 10 exposures to 1010 $\mu g/m^3$. After 20 exposures to 1010 $\mu g/m^3$ the bactericidal activity, although not significantly, was depressed. The significant increases after exposures to 1010 but not to 2040 $\mu g/m^3$ may be the result of having again reached the no-effect level where more pronounced and consistent trends could be demonstrated only through additional replicate studies.

In other studies we have often observed increased bactericidal activity in lungs of mice exposed to aerosols that had no other apparent significant health effects, whereas exposures to aerosols that produced detrimental health effects resulted in depressed bactericidal activity. It appears that the increases may be due to activation of the AM by having phagocytized the inhaled dust. On the other hand, inhalation of toxic particles, and perhaps sufficiently high concentrations of inert particles appear to overcome this initial activation and increase, and possibly depresses the bactericidal capacity of the macrophages in the lungs.

The results of these *in vivo* studies have shown no differences in the pulmonary free cells lavaged from the lungs of mice which could be related to the exposures. Inhalation of the copper smelter dust caused significant increases in susceptibility to respiratory streptococcal infection and decreases in pulmonary bactericidal activity in lungs. Coal fly ash No. 2 did not have this effect at comparable exposure conditions.*

The data clearly demonstrate that the copper smelter dust was not only more cytotoxic to AM *in vitro* but had more significant deleterious effects in inhalation exposures *in vivo* than the fluidized-bed coal fly ash under the same experimental conditions. Thus the major objective of these studies has been accomplished by demonstrating that the potential inhalation hazard of a particulate substance can be predicted on the basis of the *in vitro* RAM screening assay.

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* Coal fly ash No. 4 was examined *in vivo* in another study supported by the EPA under Grant No. R805317 and conducted in our laboratory to compare the effects of inhalation exposure to a coal- and oil-fired power plant fly ash. The data, that will be summarized in a separate report, indicate that inhalation of fly ash No. 4 produced more significant changes in the host defenses of mice than fly ash No. 2, and the effects were fewer and less pronounced than after inhalation of the copper smelter dust. Thus these *in vivo* observations are an agreement with the *in vitro* data described in the present report demonstrating an intermediate toxicity ranking for fly ash No. 4.

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